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14. ABSTRACT
As a result of androgen ablation TGF-ß1 expression levels transiently elevate and regression of benign prostate hyperplasia as well asprostate cancer cells for the most part occur. Better understanding of prostate androgen responsiveness is critical in understanding andultimately combating androgen-non-responsive prostate cancer. Studying the conditional TGF-ß type II receptor fibroblast knockoutmouse model we developed (Tgfbr2fspko), we found that TGF-ß signaling in the prostate stromal fibroblasts regulate both stromal andepithelial differentiation in the prostate. Notably the data dispels previous reports that TGF-ß signaling is required for myofibroblastdifferentiation. As proposed we attempted to develop mice that are stromally knocked out for TGF-ß signaling and express the large Tantigen in the prostate epithelia, but was unsuccessful. We have however acquired techniques in our laboratory to perform tissuerecombination experiments where the identical cell types (prostate stroma and epithelia) can generate prostate glands throughxenografting, that display similar phenotypic characteristics of intact mice. We hope to gain permission to progress with theseexperiments in order to address the mechanism of stromal TGF-ß signaling impact on prostate cancer androgen responsiveness.

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a. Introduction

The prostate epithelial and stromal compartments interact to regulate prostate development and function, in part through the tight regulation of glandular apoptosis and proliferation. These interactions are mediated by various factors that include hormones and cytokines. Specific signaling by androgens is required for prostate development and maintenance of function through the stimulation of proliferation and inhibition of apoptosis of prostatic epithelial cells (Hayward and Cunha, 2000; Montgomery et al., 2001). Cytokines such as EGF, IGF, and TGF-ß isoforms can also in-turn stimulate the expression of the androgen receptor (AR) in an androgen- independent fashion (Byrne et al., 1996; Culig et al., 1996). Often the development and progression of prostate cancer is dependent on androgens and their receptor for prostate cellular proliferation and differentiation. As a result, its inhibition has been the primary therapy for metastatic prostate cancer and much effort has been devoted to elucidating the role of the androgen receptor in prostate cancer. As a result of androgen ablation, TGF-ß1 expression levels transiently elevate and regression of benign prostate hyperplasia as well as prostate cancer cells for the most part occur. Better understanding of prostate androgen responsiveness is critical since androgen-signaling antagonists are currently used in treating patients with malignant prostate cancer, benign prostate hyperplasia, and as a chemoprevention of prostate cancer in clinical trials. However, populations of hormone-non-responsive cancer cells unfortunately frequently arise. The central hypothesis of this proposal is that TGF-ß signaling in the prostatic fibroblasts contributes to normal prostatic epithelial differentiation; when this signal is altered in the case of some cancers the differentiation status of the epithelia is altered.

TGF-ß isoforms (TGF-ß1, ß2, ß3) have long been established as physiological regulators of prostate growth because of their ability to inhibit cell proliferation and mediate apoptosis (Kyprianou and Isaacs, 1989; Martikainen et al., 1990). TGF-ßs exert their effects through binding to the TGF-ß type II receptor (TßRII) and subsequent recruitment of the type I receptor (TßRI) for downstream cytoplasmic signaling through multiple parallel signaling pathways (Attisano and Wrana, 2002). TGF-ß plays a key role in the steroidal regulation of tissues and in the important growth regulation axis existing between androgenic signaling, smooth muscle differentiation and epithelial proliferation. The present proposal seeks to address the role of TGF-ß signaling in mouse prostate tissue under in vivo conditions in light of signaling pathways identified through studies in cell lines. In order to understand the role of TGF-ß in the prostate we have developed and studied mouse models, that employ the Cre-lox methodology to conditionally ablate TßRII expression in the stromal fibroblasts (Bhowmick et al., 2004) and those that express the large SV40 T antigen transgene (TAG) in the prostate epithelia (from collaborator, Dr. Robert Matusik, Vanderbilt U., TN) (Kasper et al., 1998). The proposal was based on preliminary data that ablation of TßRII in fibroblasts results in preneoplastic prostate intraepithelial neoplasia (PIN) lesions and prostate-specific TAG expression results in PIN and progression of focal adenocarcinoma (Kasper et al., 1998). We proposed to develop mice that expressed both TAG in the prostate epithelia and concomitant loss of TßRII in the stromal fibroblasts (mouse model termed TNT) to examine epithelial and stromal differentiation (Task1). Since these TNT mice were not thought not to be able to live past 8 weeks of age, we also proposed to rescue the prostatic tissues from these mice as xenografts and further study differences in cellular differentiation and androgen responsiveness (Task2).

In the last annual report, we described the importance of Wnt signaling in the prostatic epithelia to support its androgen independent survival. In summary, we found that the loss of TGF-ß responsiveness of the prostatic stroma resulted in the upregulation of Wnt ligand expression that mediated paracrine signaling in the epithelial compartment. We have furthered these findings at the clinical and mechanistic level in the past year.

b. Body

Human prostate cancer is associated with the loss of the TGF-ß type II receptor expression the stromal compartment

We wanted to take a closer look at the stromal compartment for the expression of TGF-ß type II receptor (TßRII) in human prostate tissues. Immunohistochemistry of 59 patient tissues suggested majority of benign prostate epithelia and stroma was in fact positive for TßRII expression (**Figure 1**). However in both Gleason 7 and 9 tumors examined most of the cancer associated stromal cells were devoid of TßRII expression. Interestingly, most of the epithelia in all tissues examined were highly positive for histochemical staining. In the past, the loss of the TGF-ß receptor has been only reported in the epithelial compartment of highly aggressive prostate cancer. We are still looking into acquiring more cancer, benign, as well as PIN tissue to further support these findings. This result provides support to the mouse model we developed earlier, that had a loss of TßRII expression in the stromal compartment developing PIN lesions ($Tgfbr2^{fspKO}$).

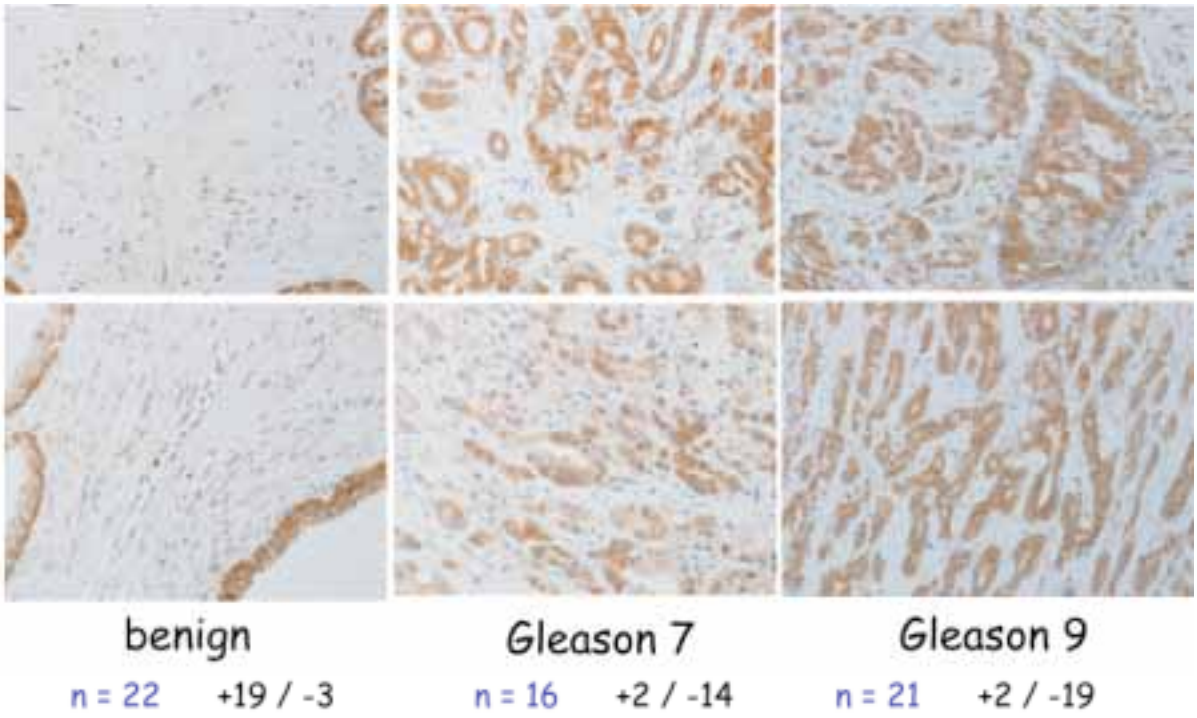

Figure 1. Immunohisto-chemistry for the expression of TßRII. Specific attention was given to TßRII expression (brown) in the stromal compartment in the prostatic tissues. All sections were nuclear counterstained with hematoxylin (blue).

The loss of TGF-ß type II receptor expression in the prostatic stroma can lead to adenocarcinoma in mice

The $Tgfbr2^{fspKO}$ mice are generally frail mice that die by 6 weeks of age. The short age of the mice did not allow for us to observe the progression of the prostatic phenotype. However, through the subrenal grafting technique described previously, we were able to rescue the prostates of $Tgfbr2^{fspKO}$ mice into both immunocompromised SCID and syngenic C57/Bl6 mice. Following seven months of xenografting, we found that the prostates of $Tgfbr2^{fspKO}$ mice had developed adenocarcinoma phenotype (**Figure 2)**. The prostates of the control, $Tgfbr2^{floxE2/floxE2}$, grafted under the same conditions maintained a normal phenotype.

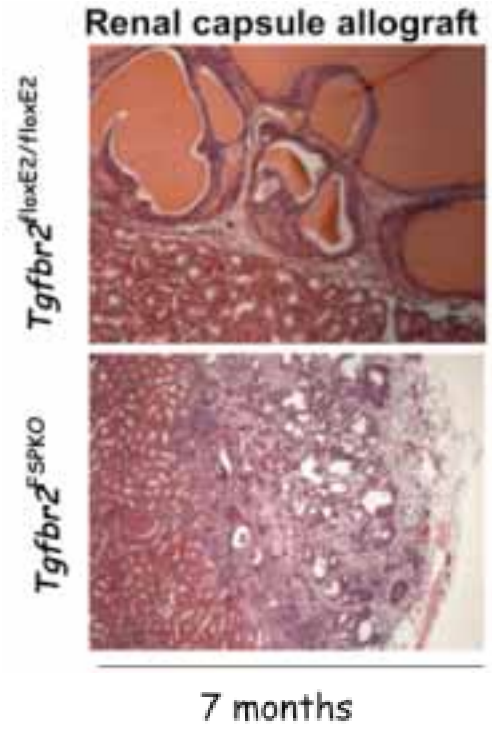

Figure 2. The progression of tumorigenesis in Tgfbr2^{fspKO} prostates to adenocarcinoma.

The greater proliferative potential of the prostate mediated by the Tgfbr2^{fspKO} stromal cells is not unique to the TNT model

The original proposal suggested the genetic recombination of the loss of the TßRII expression in the stromal compartment with the overexpression of the large T antigen in the epithelial compartment to be termed the TNT model (described in the previous report). However, manuscript reviewers suggested the need for other examples of the paracrine role for the stromal deletion of *Tgfbr2*. we addressed this concern through the development of sub-renal capsule tissue recombination models of the Tgfbr2^{fspKO} prostatic stromal cells with the well established human prostate cancer cell line, LNCaP cells. the recombination of the LNCaP cells with Tgfbr2^{fspKO} stromal cells resulted in greater tumor size compared to when grafted with control, Tgfbr2$^{floxE2/floxE2}$ stromal cells (n =12, **Figure 3A**). As the LNCaP cells are adenocarcinoma cells little histologic differences were observed between the association with either stromal cell type (**Figure 3B**). However, the greater tumor size was clearly a result of increased proliferation, as determined by phospho-histone3 immunohistochemical staining (**Figure 3B**). The Tgfbr2^{fspKO} associated tumors were also refractile to androgen ablation (data not shown).

A

B

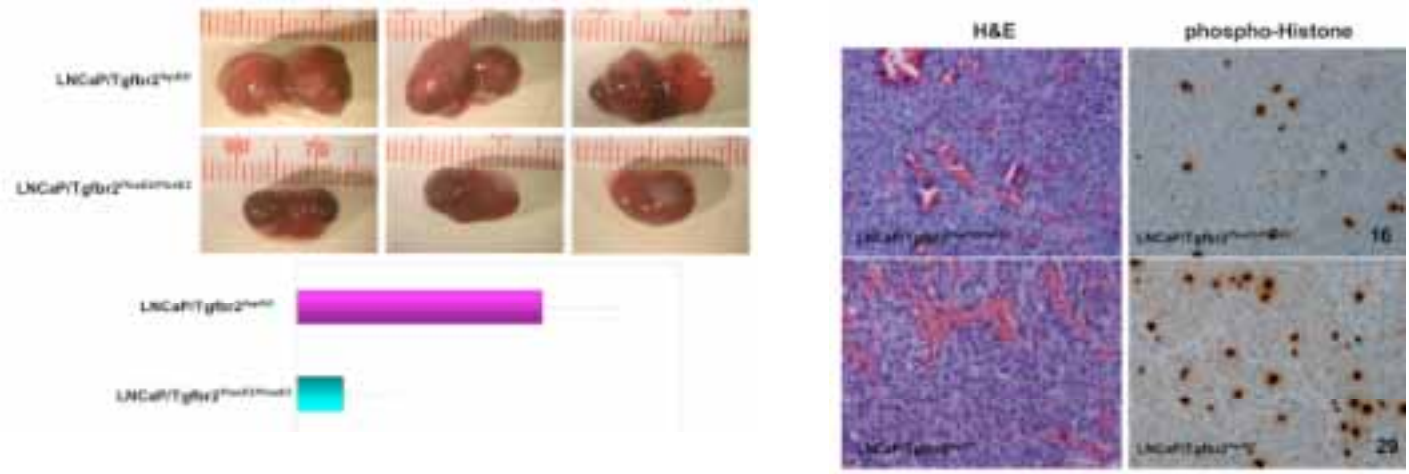

Figure 3. The tissue recombination of LNCaP and prostatic stromal cells from Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} mice. (A) Gross tumor size of recombinations associated with Tgfbr2^{fspKO} stromal cells was 5 times greater than those associated with Tgfbr2$^{floxE2/floxE2}$ cells. (B) The histology of the tumors were not significantly different, however the proliferation rate was significantly greater, based on the counting phosphorylated-histone 3 positive cells in 20 high power fields - mean indicated in the right corner.

TGF-ß in the prostatic stroma

which the tumorigenic phenomena observed in ografts. In the previous report, we described the lls from Tgfbr2^{fspKO} mice. The neutralization of able to reverse the androgen independence of the TßRII of the stroma. In the subsequent studies, on was consistently up regulated close to 10 fold those from Tgfbr2$^{floxE2/floxE2}$ mice (**Figure 4A**). ^{fspKO} prostatic stromal cells, although to a lesser on tumor growth. This was initially done *in vitro*, onto LNCaP cells causing the cells to proliferate urve was observed as increasing concentrations of Tgfbr2^{fspKO}–conditioned media (**Figure 4B**). Cell following treatment with conditioned media with he over expression of Wnt3a by the Tgfbr2^{fspKO} sm for increased prostatic growth in the presence

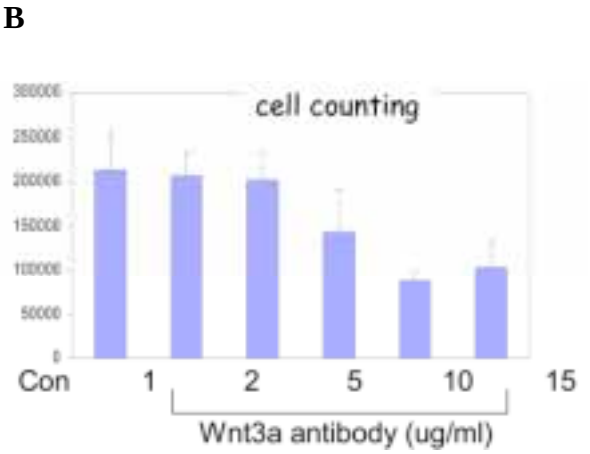

Figure 5. Wnt3a expression by Tgfbr2^{fspKO} prostatic stromal cells mediates greater tumor growth. (A) RNA from Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} cells were subjected to realtime quantitative RT-PCR for seventeen Wnt ligand isoforms. (B) Conditioned media from Tgfbr2^{fspKO} stromal cells were placed on LNCaP cells in the absence and presence of an increasing concentration of Wnt3a neutralizing antibody. The LNCaP cells were counted following 72hs of incubation as a measure of proliferative measure of the cells.

Epithelial TGF-ß responsiveness does not affect prostatic regression following androgen ablation

To study the role of TGF-ß on the prostatic epithelia following androgen ablation, we developed a conditional *Tgfbr2* knockout by crossing NKX3.1-Cre mice with Tgfbr2$^{floxE2/floxE2}$ mice [1], termed Tgfbr2$^{NKX3.1KO}$. Crossing the Tgfbr2$^{NKX3.1KO}$ mice further into the Rosa26 line enabled the immunohistochemical localization for ß-galactosidase expression associated with recombination in the prostatic epithelia (**Figure 5A**). However, since Tgfbr2$^{NKX3.1KO}$ mice died at birth, the prostatic development and androgen responsiveness could not be studied. Consequently, their urogenital sinuses were rescued by allografting into male syngeneic, C57/Bl6 mice. Six weeks following allografting to the sub-renal capsule, as above, the host mice were either left intact or

castrated. All grafts were harvested 7 weeks following allografting for histologic examination. Hematoxylin and eosin staining revealed little difference in the ductal structures of the Tgfbr2$^{NKX3.1KO}$ prostates compared to Tgfbr2$^{floxE2/floxE2}$ controls (**Figure 5B**). TUNEL staining of the allografts suggested greater apoptosis of Tgfbr2$^{NKX3.1KO}$ prostates (both stromal and epithelial compartments) in the intact hosts compared to Tgfbr2$^{floxE2/floxE2}$ allografts. In turn, observed greater epithelial proliferation by Ki67 staining in the Tgfbr2$^{NKX3.1KO}$ prostates in intact host mice, was an indication of the elevated intrinsic turnover of Tgfbr2$^{NKX3.1KO}$ prostates (data not shown). However, there was further elevation of prostatic epithelial TUNEL staining of the Tgfbr2$^{NKX3.1KO}$ prostates following castration, similar to Tgfbr2$^{floxE2/floxE2}$ allografts (**Figure 5B**). Taken together, there was no apparent difference in prostatic apoptosis observed between Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2$^{NKX3.1KO}$ prostates following castration.

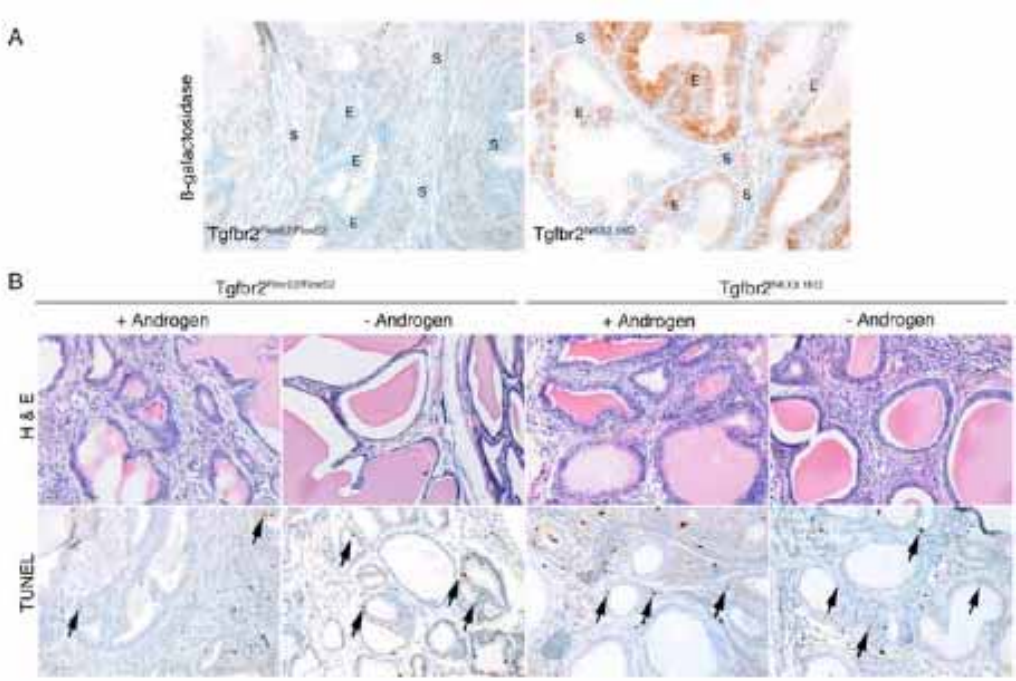

Figure 5. Conditional knockout of *Tgfbr2* in the prostatic epithelia, Tgfbr2$^{NKX3.1KO}$, did not significantly affect the response to androgen ablation compared to control Tgfbr2$^{floxE2/floxE2}$ prostates. (**A**) Immunohistochemistry for ß-galactosidase expression in Tgfbr2$^{NKX3.1KO}$/Rosa26 indicates Cre-recombination in the prostatic epithelia compared to control, Tgfbr2$^{floxE2/floxE2}$/Rosa26 prostates with no detectible staining (brown). The stromal (S) and epithelial (E) compartments are indicated. (**B**) Hematoxylin and eosin staining of the upper panels suggest similar development of Tgfbr2$^{NKX3.1KO}$ and Tgfbr2$^{floxE2/floxE2}$ prostates (n=4). However, TUNEL staining in the lower panels indicate differential apoptosis of the prostatic epithelia of Tgfbr2$^{NKX3.1KO}$ and Tgfbr2$^{floxE2/floxE2}$ allografts in hosts that were not castrated (+Androgen) and castrated (-Androgen). The immunohistochemistry stained sections (A, B) were nuclear counterstained with hematoxylin (blue).

c. Significance: Surgery and androgen ablation therapy remains the major treatment for prostate cancer. However, within a year of treatment >80% of prostate cancer becomes androgen independent as a result of documented mutations in AR and unknown factors. New treatments are required that are more effective irrespective of the structure of the AR in the cancerous epithelial cells. Based on our analysis of the $Tgfbr2^{fspko}$ mouse model and human prostate cancer tissues, we believe that targeting therapy to the prostate stroma rather than the cancer cells may be prudent in patients' androgen non-responsive prostate cancer. The studies in the past year suggest that the specific treatment may be through the targeting of the Wnt3a signaling axis. The non-transformed stromal cells are likely to have greater genomic stability and are less likely to be subject of mutation adaptation since the target would be the secretion of paracrine factors that would act on the epithelia.

d. Plans: The primary experimental task would be to follow up the results presented with neutralizing antibody treatment of prostate tumors in mouse models *in vivo*. Preliminary data not shown suggest good tolerance of the neutralizing antibody in mice. We will be able to assess the impact of the Wnt3a neutralizing antibody on tumor size within 6 months. The other immediate goal is to have the two manuscripts currently in review to be finally published in high level journals. Finally, we would like to leverage the findings of this grant to apply for a NIH supported R01.

KEY RESEARCH ACCOMPLISHMENTS

- Identified for the first time, that human prostate cancer often has a loss of TßRII expression in the stromal compartment . Thepithelial compartment, in contrast rarely looses TßRII expression except in foci of very high grade prostate cancer.
- We showed that Wnt3a is highly expressed by the prostatic stroma and that its neutralization can inhibit prostate cancer growth *in vitro*
- The loss of TßRII expression in the epithelial compartment does not significantly affect androgen responsivity of the prostate.

REPORTABLE OUTCOMES

Research
Manuscripts

Veronica R. Placencio, Ali-Reza Sharif-Afshar, Xiaohong Li, Hongxia Huang, Consolate Uwamariya, Eric G. Neilson, Michael M. Shen, Simon W. Hayward, Neil A. Bhowmick (2007) TGF-ß responsiveness modulates paracrine prostatic sensitivity to androgen ablation. *In review.*

Xiaohong Li, Veronica R. Placencio, Juan Iturregui, Ali-Reza Afshar-Sharif, Neil A. Bhowmick (2007) Loss of TGF-ß receptor expression in the prostatic stroma can promote tumorigenesis in men. *In review*

Abstracts

Veronica R. Placencio, Ali-Reza Sharif-Afshar, Xiaohong Li, Robert J. Matusik, Simon W. Hayward, Neil A. Bhowmick (2006) Wnt signaling contributes to the androgen independence of prostatic epithelia resulting from the loss of TGF-ß responsiveness of the stroma. Annual Fall Meeting of the Society for Basic Urologic Research. Pheonix, AZ.

Awards received based on work supported by this grant

Invited speaker, Travel Award to the Annual Fall Meeting of the Society for Basic Urologic Research. Pheonix, AZ.

Products
CDNA construct, cell lines, and animal models developed

The NKX3.1-Cre mice were crossed with $Tgfbr2^{floxE2/floxE2}$ mice to generate the $Tgfbr2^{NKX3.1KO}$ mouse model. The $Tgfbr2^{NKX3.1KO}$ mice were additionally crossed with Rosa26 mice to enable visualization of cells undergoing Cre-mediated recombination.

CONCLUSION

Our study demonstrates that stromal and epithelial responsiveness to TGF-ß dictates androgen sensitivity in the epithelia of prostate glands. The mouse model with the TGF-ß type II receptor conditionally knocked out in fibroblasts, $Tgfbr2^{fspKO}$, also demonstrates the integral role of stromal TGF-ß signaling during prostatic epithelial regression [2]. The $Tgfbr2^{fspKO}$ stromal cells themselves acquired a more proliferative phenotype and promoted nearby epithelia to increase their rate of proliferation to overcome hormonal dependence. After castration of host mice, we found the prostatic ducts associated with $Tgfbr2^{fspKO}$ stromal cells were also refractile to androgen ablation. The direct role of TGF-ß signaling on the prostatic epithelia *in vivo* was minimal based on histologic differences between the $Tgfbr2^{NKX3.1KO}$ and $Tgfbr2^{floxE2/floxE2}$ prostates. However, the $Tgfbr2^{NKX3.1KO}$ prostates had greater cell turnover compared to control in non-castrated hosts. The mechanisms behind the observed phenomena highlight a paracrine TGF-ß signaling axis that is altered upon androgen ablation.

The differentiation of the prostatic epithelium and stroma occur concurrently in an androgen-dependent mechanism. The loss of stromal TGF-ß responsivity can result in the development of adenocarcinoma in the mouse model with no other directed genetic alteration in the epithelial compartment. Coincidentally, human prostate cancer also has a loss of TßRII expression in the stromal compartment. To examine the mechanism of these observations, we found that stromally derived paracrine Wnt3a mediates prostate cancer epithelial growth.

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